The influence of diet type (dairy versus intensive fattening) on the effectiveness of garlic oil and cinnamaldehyde to manipulate *in vitro* ruminal fermentation and methane production

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Abstract. The objective of this study was to evaluate the effects of increasing doses [0 (control: CON), 20, 60, 180 and 540 mg/L incubation medium] of garlic oil (GO) and cinnamaldehyde (CIN) on *in vitro* ruminal fermentation of two diets. Batch cultures of mixed ruminal microorganisms were inoculated with ruminal fluid from four sheep fed a medium-concentrate diet (MC; 50 : 50 alfalfa hay : concentrate) or four sheep fed a high-concentrate diet (HC; 15 : 85 barley straw : concentrate). Diets MC and HC were representative of those fed to dairy and fattening ruminants, respectively. Samples of each diet were used as incubation substrates for the corresponding inoculum, and the incubation was repeated on 4 different days (four replicates per experimental treatment). There were GO × diet-type and CIN × diet-type interactions ($P < 0.001$–0.05) for many of the parameters determined, indicating different effects of both oils depending on the diet type. In general, effects of GO were more pronounced for MC compared with HC diet. Supplementation of GO did not affect ($P > 0.05$) total volatile fatty acid (VFA) production at any dose. For MC diet, GO at 60, 180 and 540 mg/L decreased ($P < 0.05$) molar proportion of acetate (608, 569 and 547 mmol/mol total VFA, respectively), and increased ($P < 0.05$) propionate proportion (233, 256 and 268 mmol/mol total VFA, respectively), compared with CON values (629 and 215 mmol/mol total VFA for acetate and propionate, respectively). A minimum dose of 180 mg of GO/L was required to produce similar modifications in acetate and propionate proportions with HC diet, but no effects ($P > 0.05$) on butyrate proportion were detected. Methane/VFA ratio was reduced ($P < 0.05$) by GO at 60, 180 and 540 mg/L for MC diet (0.23, 0.16 and 0.10 mol/mol, respectively), and by GO at 20, 60, 180 and 540 mg/L for HC diet (0.19, 0.19, 0.16 and 0.08 mol/mol, respectively), compared with CON (0.26 and 0.21 mol/mol for MC and HC diets, respectively). No effects ($P = 0.16$–0.85) of GO on final pH and concentrations of NH_3-N and lactate were detected. For both diet types, the highest CIN dose decreased ($P < 0.05$) production of total VFA, gas and methane, which would indicate an inhibition of fermentation. Compared with CON, CIN at 180 mg/L increased ($P < 0.05$) acetate proportion for the MC (629 and 644 mmol/mol total VFA for CON and CIN, respectively) and HC (525 and 540 mmol/mol total VFA, respectively) diets, without affecting the proportions of any other VFA or total VFA production. Whereas for MC diet CIN at 60 and 180 mg/L decreased ($P < 0.05$) NH_3-N concentrations compared with CON, only a trend ($P < 0.10$) was observed for CIN at 180 mg/L with the HC diet. Supplementation of CIN up to 180 mg/L did not affect ($P = 0.18$–0.99) lactate concentrations and production of gas and methane for any diet. The results show that effectiveness of GO and CIN to modify ruminal fermentation may depend on diet type, which would have practical implications if they are confirmed *in vivo*.

Introduction

Identification of substances that modify rumen fermentation to increase its efficiency and decrease the amount of methane and nitrogenous pollution excreted into the environment is an important objective of current ruminant nutrition research. In the last years, the potential of a wide range of plant extracts as additives to manipulate ruminal fermentation has been extensively investigated using *in vitro* systems, but garlic oil (GO) and cinnamaldehyde (CIN) are two of the most cited extracts in the literature (Calsamiglia *et al.* 2007). Research on GO and CIN has produced, however, controversial results. In *in vitro* experiments GO and some of its compounds have been repeatedly shown to reduce the proportions of acetate and to increase those of propionate and butyrate (Busquet *et al.* 2005*a*;

Calsamiglia *et al.* 2007), but their effects on N metabolism and methane are more variable (Busquet *et al.* 2005*b*; Kamel *et al.* 2008; Kongmun *et al.* 2010). Cinnamaldehyde is the main active component of cinnamon oil, and it has been investigated because of its potential to modify N metabolism, although the results found both *in vitro* (Cardozo *et al.* 2004; Busquet *et al.* 2005*a*, 2005*b*; Benchaar *et al.* 2007) and *in vivo* (Chaves *et al.* 2008; Yang *et al.* 2010) are inconsistent. The contrasting results observed when using GO and CIN as additives to manipulate rumen fermentation could be due to many factors, including, among others, extract characteristics and dose, ruminal pH, microbial populations, and diet fed to animals or used as substrate for *in vitro* incubations. The effects of different doses of GO and CIN and of ruminal pH have been previously investigated, but to our knowledge no study has specifically addressed the influence of the diet type on the effectiveness of GO and CIN, despite the fact the diet is one of the most relevant factors affecting ruminal microbial populations (Weimer *et al.* 1999). Our hypothesis was that the effective dose and the effects of GO and CIN on *in vitro* ruminal fermentation would depend on microbial populations in the inoculum, and therefore on the type of diet fed to the host animal. This study was designed to evaluate the effects of increasing doses of GO and CIN on *in vitro* ruminal fermentation as influenced by the type of diet (i.e. dairy versus fattening), and therefore the tested diets were both fed to donor animals and incubated *in vitro*.

Materials and methods

Animals and diets

Eight rumen-cannulated Merino sheep were used as ruminal fluid donors for the *in vitro* incubations. Four sheep were fed a 50 : 50 alfalfa hay : concentrate diet (medium-concentrate diet; MC) and the other four received a 15 : 85 barley straw : concentrate diet (high-concentrate; HC). Diets were formulated to be representative of those fed to dairy animals (MC) and to growing ruminants under intensive systems of production (HC). Ingredient and chemical composition of both diets are shown in Table 1. Both diets were fed in two equal portions at 0900 and 1800 hours. Sheep were managed according to protocols approved by the León University Institutional Animal Care and Use Committee, and were adapted to the corresponding diet for 2 weeks before starting the *in vitro* incubations.

Substrates, additives and in vitro *fermentations*

Samples of the two diets fed to sheep were used as substrates for the *in vitro* fermentations. Forages and concentrates were ground through a 1-mm screen and mixed in the corresponding proportions. The two additives were supplied by Axiss France SAS (Bellegarde Sur Valserine, France). Based on previous analysis GO contained 0.65 g of diallyl disulfide, 0.15 g of diallyl trisulfide and 0.10 g of allicin per g of oil, and CIN had a 99% purity. The concentrations of each additive used were selected from previous studies (Busquet *et al.* 2005*b*; Kamel *et al.* 2008) and were: 0 (control: CON), 20 (GO20 and CIN20), 60 (GO60 and CIN60), 180 (GO180 and CIN180) and 540 (GO540 and CIN540) mg/L incubation medium, representing 0, 0.2, 0.6, 1.8 and 5.4 g/kg of the incubated substrate, respectively. Samples of substrates (300 mg DM) were weighed into 120-mL serum bottles. Additives were dissolved in ethanol the day of the incubation, and 30 μL of the corresponding solution were applied inside the bottles immediately before adding buffered rumen fluid. Control bottles received 30 μL of ethanol.

Ruminal contents were obtained immediately before the morning feeding from each sheep, mixed by diet (four sheep per diet), and strained through four layers of cheesecloth into an Erlenmeyer flask with an O_2-free headspace. Particle-free fluid was mixed with the buffer solution of Goering and Van Soest (1970; no trypticase added) in a proportion 1 : 4 (vol/vol) at 39°C under continuous flushing with CO_2.

A total of nine bottles with each substrate (one bottle per substrate and additive dose) and six bottles without substrate (blanks) were incubated. Thirty mL of buffered rumen fluid were added into each bottle under CO_2 flushing. Bottles of each substrate were filled with buffered ruminal fluid from sheep fed the same diet. Bottles were sealed with rubber stoppers and aluminium caps, incubated at 39°C, and withdrawn 16 h after inoculation (corresponding to a passage rate from the rumen of 0.06 per h). Total gas production was measured using a pressure transducer and a calibrated syringe, and a gas sample (10 mL) was removed from each bottle and stored in a Haemoguard Vacutainer (Terumo Europe N.V., Leuven, Belgium) before analysis for methane. Bottles were then uncapped, the pH was measured immediately with a pH meter, and the fermentation was slower down by swirling the bottles in ice. One mL of content was added to 1 mL of deproteinising solution [i.e. metaphosphoric acid (20 g/L) and crotonic acid (4 g/L)] for volatile fatty acid (VFA) determination, 1 mL was added to 1 mL 0.5 M HCl for NH_3-N

Table 1. Ingredient composition and chemical analysis of medium- (MC) and high-concentrate (HC) diets fed to donor sheep and used as substrates for the *in vitro* fermentations

Ingredient (g/kg of DM)	Diet	
	MC	HC
Alfalfa hay	500	–
Barley straw	–	150
Barley	199	425
Corn	96.0	255
Soybean meal	71.0	150
Lupin	60.0	–
Oat	31.5	–
Fullfat soybean	15.0	–
Calcium carbonate	6.9	6.7
Sugarcane molasses	5.0	–
NaCl	3.5	3.7
Dicalcium phosphate	2.1	–
Mineral/vitamin premix[A]	10.0	10.0
Chemical analyses (g/kg of DM)		
Organic matter	935	942
Crude protein	186	150
Neutral detergent fibre	394	357
Acid detergent fibre	179	126

[A]Declared composition (g/kg mineral/vitamin premix): vitamin A, 600 000 IU; vitamin D_3, 120 000 IU; vitamin E, 1 g; vitamin B_1, 33 mg; niacine, 1.5 g; S, 5 g; IK, 300 mg; SO_4Fe, 1 g; ZnO, 4 g; MnO, 2 g; $CoSO_4$, 60 mg; Na_2SeO_3, 30 mg; Ethoxyquin, 30 mg.

analysis, and 1 mL was immediately frozen for lactate analysis. The experiment was repeated on four non-consecutive days to get four bottles per experimental treatment.

Analytical procedures

Dry matter (ID 934.01), ash (ID 942.05) and N (ID 984.13) were determined according to the Association of Official Analytical Chemists (1999). Neutral detergent fibre with heat-stable amylase and expressed inclusive of residual ash (NDF) and acid detergent fibre expressed inclusive of residual ash (ADF) analyses were carried out according to Van Soest *et al.* (1991) using an ANKOM^{220} Fibre Analyser unit (ANKOM Technology Corporation, Fairport, NY, USA). Sodium sulfite and heat-stable amylase were used in analysis of NDF and ADF, and they were expressed inclusive of residual ash. Concentrations of VFA, NH_3-N and total lactate were determined as described by García-Martínez *et al.* (2005), and methane was analysed by gas chromatography following the procedure described by Martínez *et al.* (2010).

Calculations and statistical analyses

The amounts of VFA produced in each bottle were calculated by subtracting the amount present initially in the incubation medium from that determined at the end of the incubation period. The volume of gas produced was corrected for temperature (273 K) and pressure (1.013×10^5 Pa), and the amount of methane (mmol) was calculated by multiplying gas produced by the concentration of methane in the analysed sample. The gas produced was calculated as the sum of the gas measured plus the gas in the head space of the cultures. Recovery of hydrogen in each bottle was estimated from net productions of acetate, propionate, butyrate, valerate and methane (Demeyer 1991), and the amount of organic matter apparently fermented (AFOM) was estimated from VFA production using the equation proposed by Demeyer and Van Nevel (1975).

Differences between the two inocula were assessed by ANOVA with inoculum and inoculum × incubation day as fixed effects and incubation day as a random effect. *In vitro* data were analysed separately for each additive. Five concentrations of additive (0, 20, 60, 180 and 540 mg/L), two diets (MC and HC), and the interaction of additive dose × diet type were included in the model as fixed effects, whereas incubation day was considered as a random effect. Nonorthogonal polynomial contrasts were used to test for linear, quadratic, and cubic effects of additives. The PROC MIXED procedure of SAS (2004) was used for all statistical analyses. Significance was declared at $P < 0.05$, whereas $P < 0.10$ values were considered to be a trend. When a significant effect of additive dose or an additive dose × diet-type interaction was detected, each additive dose mean was compared with the corresponding control by Dunnett test. Within each additive dose and diet type, there were four values for each of the measured variables.

Results

There were marked differences between the fermentation characteristics of the two inocula used in this experiment (Table 2). The inoculum from MC-fed sheep had greater pH ($P = 0.03$), NH_3-N concentrations ($P = 0.03$), acetate proportion ($P = 0.002$) and acetate : propionate ratio ($P = 0.006$), but lower propionate proportion ($P = 0.02$) compared with the inoculum from sheep fed the HC diet. In contrast, there were no differences between both inocula either in the molar proportions of butyrate ($P = 0.75$), other VFA ($P = 0.98$) or in the concentrations of lactate ($P = 0.53$). Initial pH in the incubation medium was 6.98 and 6.59 for MC and HC diets, respectively (results not shown).

Garlic oil effects

Effects of supplementing batch cultures with GO on rumen fermentation variables are shown in Tables 3 and 4. Diet type affected ($P < 0.001$) all parameters measured, with the exception of total lactate concentrations ($P = 0.11$). Garlic oil × diet-type interactions ($P < 0.001$–0.003) were detected for molar proportions of acetate, butyrate and caproate, production of gas and methane and acetate : propionate and methane : VFA ratios. Increasing levels of GO linearly decreased acetate ($P < 0.001$) and caproate ($P = 0.01$) proportions and acetate : propionate ratio ($P < 0.001$), but increased ($P < 0.001$) propionate, butyrate and valerate proportions. The dose required to detect a significant effect was generally lower for the MC diet than for the HC one. Increased amounts of GO linearly decreased ($P < 0.001$) the production of gas and methane. As a consequence of these changes, methane : VFA ratio was also reduced ($P < 0.001$) when increasing GO level. Proportion of methane in the gas produced and hydrogen recovery were also linearly reduced ($P < 0.001$) as GO levels increased.

Table 2. Mean values of pH and concentrations of volatile fatty acids (VFA), NH_3-N and total lactate in ruminal fluid used as inoculum for the *in vitro* incubations

Diet[A]	pH	Rumen fluid							
		Total VFA (mmol/L)	Acetate (mmol/mol total VFA)	Propionate (mmol/mol total VFA)	Butyrate (mmol/mol total VFA)	Other VFA[B] (mmol/mol total VFA)	Acetate : propionate (mol/mol)	Ammonia-N (mg/L)	Total lactate (mg/L)
MC	6.73	117	654	180	109	56.4	3.63	127	8.98
HC	6.45	99	585	226	132	56.6	2.60	114	8.40
s.e.m.	0.073	5.79	6.5	10.3	3.8	8.56	0.142	3.2	0.091
P-value	0.03	0.05	0.002	0.02	0.75	0.98	0.006	0.03	0.53

[A]Diets fed to donor sheep. Diet MC (medium-concentrate) was composed of alfalfa hay and concentrate (500 and 500 g/kg DM, respectively) and diet HC (high-concentrate) was composed of barley straw and concentrate (150 and 850 g/kg DM, respectively).

[B]Calculated as the sum of isobutyrate, isovalerate, valerate and caproate.

Table 3. Effects of five doses of garlic oil (0, 20, 60, 180 and 540 mg/L for CON, GO20, GO60, GO180 and GO540, respectively) on total volatile fatty acid production (VFA), molar proportions of VFA, acetate : propionate ratio, and apparently fermented organic matter (AFOM) after *in vitro* fermentation of diets (300 mg) with medium-concentrate (MC) and high-concentrate (HC) content by mixed rumen microorganisms for 16 h (n = 4)

L, linear effect of GO dose; Q, quadratic effect of GO dose

Variable	Diet	Treatment					s.e.m.	Statistical significance of the effects (P-value)			
		CON	GO20	GO60	GO180	GO540		GO		Diet	GO × diet
								L	Q		
Total VFA (mmol)	MC	2.07	2.07	2.08	1.99	1.93	0.057	0.26	0.09	<0.001	0.46
	HC	2.20	2.26	2.23	2.18	2.20	–	–	–	–	–
					Molar proportion (mmol/mol total VFA)						
Acetate	MC	629	625	608[A]	569[A]	547[A]	5.3	<0.001	<0.001	<0.001	<0.001
	HC	525	533	529	508[A]	479[A]	–	–	–	–	–
Propionate	MC	215	218	233[A]	256[A]	268[A]	6.0	<0.001	0.003	<0.001	0.06
	HC	309	306	309	326[A]	350[A]	–	–	–	–	–
Butyrate	MC	106	108	112[A]	124[A]	138[A]	2.9	<0.001	0.002	<0.001	<0.001
	HC	140	135	135	140	145	–	–	–	–	–
Isobutyrate	MC	12.5	11.5	10.8	12.5	13.4	3.46	0.94	0.35	<0.001	0.81
	HC	3.3	2.9	3.1	3.0	3.3	–	–	–	–	–
Isovalerate	MC	13.5	13.5	13.2	14.0	12.7	0.52	0.37	0.91	<0.001	0.55
	HC	6.9	7.3	7.5	7.2	6.4	–	–	–	–	–
Valerate	MC	17.5	17.7	18.3	19.3[A]	19.1[A]	0.46	<0.001	0.06	<0.001	0.56
	HC	13.3	13.3	13.8	14.7[A]	15.5[A]	–	–	–	–	–
Caproate	MC	6.8	6.2	7.1	4.8[A]	1.5[A]	0.53	0.01	0.05	<0.001	<0.001
	HC	2.7	2.6	2.7	2.2	1.5[A]	–	–	–	–	–
Acetate : propionate (mol/mol)	MC	2.93	2.87	2.62[A]	2.23[A]	2.05[A]	0.66	<0.001	<0.001	<0.001	<0.001
	HC	1.70	1.75	1.71	1.57[A]	1.37[A]	–	–	–	–	–
AFOM (mg)	MC	177	178	180	173	171	5.5	0.49	0.16	<0.001	0.81
	HC	198	203	200	196	200	–	–	–	–	–

[A]For each diet and variable, means differ from CON ($P < 0.05$).

Table 4. Effects of five doses of garlic oil (0, 20, 60, 180 and 540 mg/L for CON, GO20, GO60, GO180 and GO540, respectively) on final pH, concentrations of NH_3-N and total lactate, production of gas and methane, methane/volatile fatty acid ratio (methane/VFA), proportion of methane in the gas produced and hydrogen recovery after *in vitro* fermentation of diets (300 mg) with a medium- (MC) and high- (HC) concentrate content by mixed rumen microorganisms for 16 h (n = 4)

L, linear effect of GO dose; Q, quadratic effect of GO dose

Variable	Diet	Treatment					s.e.m.	Statistical significance of the effects (P-value)			
		CON	GO20	GO60	GO180	GO540		GO		Diet	GO × diet
								L	Q		
pH	MC	6.63	6.66	6.64	6.67	6.65	0.027	0.16	0.89	<0.001	0.25
	HC	6.51	6.52	6.53	6.54	6.53	–	–	–	–	–
NH_3-N (mg/L)	MC	232	228	231	243	230	8.8	0.86	0.98	<0.001	0.85
	HC	95	99	93	91	90	–	–	–	–	–
Total lactate (mg/L)	MC	13.0	9.8	9.9	9.6	10.5	1.78	0.12	0.40	0.11	0.57
	HC	9.9	10.1	9.0	9.4	7.5	–	–	–	–	–
Gas (mmol)	MC	3.22	3.22	3.24	3.03[A]	2.90[A]	0.047	<0.001	0.001	<0.001	0.002
	HC	3.35	3.36	3.34	3.25[A]	3.26[A]	–	–	–	–	–
Methane (mmol)	MC	0.54	0.53	0.47[A]	0.32[A]	0.20[A]	0.022	<0.001	<0.001	<0.001	0.001
	HC	0.47	0.42[A]	0.42[A]	0.35[A]	0.18[A]	–	–	–	–	–
Methane/VFA (mol/mol)	MC	0.26	0.26	0.23[A]	0.16[A]	0.10[A]	0.012	<0.001	0.006	<0.001	0.003
	HC	0.21	0.19[A]	0.19[A]	0.16[A]	0.08[A]	–	–	–	–	–
Proportion of methane (mmol/mol gas)	MC	168	164	146[A]	104[A]	68[A]	6.7	<0.001	0.003	<0.001	0.003
	HC	140	126[A]	127[A]	107[A]	55[A]	–	–	–	–	–
Hydrogen recovery (%)	MC	90.2	89.7	85.5	75.6[A]	65.7[A]	2.61	<0.001	0.16	0.02	0.06
	HC	92.2	84.4[A]	87.8	83.8[A]	71.2[A]	–	–	–	–	–

[A]For each diet and variable, means differ from CON ($P < 0.05$).

Neither linear nor quadratic effects (P = 0.09–0.98) of GO supplementation were observed for total VFA production, isobutyrate and isovalerate proportions, AFOM, final pH, and concentrations of NH_3-N and lactate.

Cinnamaldehyde effects

Effects of CIN on *in vitro* rumen fermentation are shown in Tables 5 and 6. Diet type affected (P < 0.001–0.008) most of parameters measured, with the exception of AFOM (P = 0.07), total lactate concentration (P = 0.08) and hydrogen recovery (P = 0.99). Cinnamaldehyde × diet-type interactions occurred (P<0.001–0.04) for all the parameters studied, with the exception of pH (P = 0.15) and proportions of caproate (P = 0.77), isobutyrate (P = 0.98) and isovalerate (P = 0.59). Increasing levels of CIN linearly increased (P = 0.002) acetate proportion and decreased propionate proportion (P = 0.04) and NH_3-N concentrations (P < 0.001). Neither linear nor quadratic effects (P = 0.10–0.99) of CIN supplementation were detected for production of total VFA, gas and methane, proportions of butyrate, isobutyrate, valerate, isovalerate and caproate, AFOM, final pH, and concentrations of lactate.

Discussion

This study was specifically designed to examine the effects of increasing doses of GO and CIN on *in vitro* ruminal fermentation as influenced by the type of diet (dairy versus fattening), and the differences in fermentation parameters observed between the inocula indicate the existence of different microbial communities promoted by feeding the two diets to donor sheep. Values of the main fermentation parameters are in agreement with fermentation patterns in sheep fed similar diet types (Carro *et al.* 2000; Ramos *et al.* 2009).

Garlic oil effects

Supplementation of GO did not decrease total VFA production with any diet, suggesting that ruminal fermentation was not inhibited even at the highest dosage (540 mg/L). Similar to our results Busquet *et al.* (2006) observed that GO at 300 mg/L did not affect total VFA production in batch cultures and a 50 : 50 forage : concentrate diet as substrate, although at 3000 mg/L decreased (P < 0.05) VFA production to 86% of that in CON cultures. Volatile fatty acids represent the main supply of metabolisable energy for ruminants, and therefore a reduction in their production would be nutritionally unfavourable for the host animal (Busquet *et al.* 2006) and should be avoided. The interactions GO × diet type observed for some of the parameters determined could be due to different microbial communities present in the two inocula. In general, effects of GO were more pronounced for MC compared with HC diet, which would indicate that the effects of GO are diet-dependent.

In agreement with our results, other *in vitro* studies using batch cultures and a 50 : 50 forage : concentrate diet (Busquet *et al.* 2005*a*, 2006) or continuous culture fermenters fed a 30 : 70 forage : concentrate diet (Busquet *et al.* 2005*b*) demonstrated

Table 5. Effects of five doses of cinnamaldehyde (0, 20, 60, 180 and 540 mg/L for CON, CIN20, CIN60, CIN180 and CIN540, respectively) on total volatile fatty acid production (VFA), molar proportions of VFA, acetate : propionate ratio, and apparently fermented organic matter (AFOM) after *in vitro* fermentation of diets (300 mg) with a medium- (MC) and high- (HC) concentrate content by mixed rumen microorganisms for 16 h (n = 4)

L, linear effect of CIN dose; Q, quadratic effect of CIN dose

Variable	Diet	Treatment					s.e.m.	Statistical significance of the effects (P-value)			
		CON	CIN20	CIN60	CIN180	CIN540		CIN		Diet	CIN × diet
								L	Q		
Total VFA (mmol)	MC	2.07	2.09	2.09	2.12	1.87[A]	0.070	0.22	0.30	<0.001	<0.001
	HC	2.20	2.20	2.15	2.29	1.26[A]	–	–	–	–	–
Molar proportion (mmol/mol total VFA) of:											
Acetate	MC	629	625	628	644[A]	580[A]	7.3	0.002	0.06	<0.001	<0.001
	HC	525	526	533	540[A]	554[A]	–	–	–	–	–
Propionate	MC	215	219	218	204	220	6.2	0.04	0.09	<0.001	<0.001
	HC	309	310	305	301	272[A]	–	–	–	–	–
Butyrate	MC	106	105	104	104	148[A]	2.9	0.10	0.63	<0.001	<0.001
	HC	140	138	137	136	139	–	–	–	–	–
Isobutyrate	MC	12.5	13.0	12.5	10.7	11.2	2.84	0.40	0.53	<0.001	0.98
	HC	3.3	3.1	3.1	1.6	3.8	–	–	–	–	–
Isovalerate	MC	13.5	13.4	13.4	12.5	12.4	0.56	0.27	0.38	<0.001	0.59
	HC	6.9	6.9	7.1	6.9	6.8	–	–	–	–	–
Valerate	MC	17.5	17.9	17.6	17.3	20.2[A]	0.95	0.46	0.74	<0.001	0.02
	HC	13.3	13.1	12.9	12.7	19.8[A]	–	–	–	–	–
Caproate	MC	6.8	6.9	7.1	7.4	8.5	0.69	0.73	0.94	<0.001	0.77
	HC	2.7	2.5	2.6	2.3	4.6	–	–	–	–	–
Acetate : propionate (μmol/μmol)	MC	2.93	2.86	2.88	3.17[A]	2.64[A]	0.097	0.02	0.04	<0.001	<0.001
	HC	1.70	1.70	1.75	1.79	2.04[A]	–	–	–	–	–
AFOM (mg)	MC	177	178	178	181	166	6.5	0.28	0.30	0.07	<0.001
	HC	198	198	194	207	113[A]	–	–	–	–	–

[A]For each diet and variable, means differ from CON (P < 0.05).

Table 6. Effects of five doses of cinnamaldehyde (0, 20, 60, 180 and 540 mg/L for CON, CIN20, CIN60, CIN180 and CIN540, respectively) on final pH, concentrations of NH_3-N and total lactate, production of gas and methane, methane/volatile fatty acid ratio (methane/VFA), proportion of methane in the gas produced and hydrogen recovery after *in vitro* fermentation of diets (300 mg) with a medium- (MC) and high- (HC) concentrate content by mixed rumen microorganisms for 16 h (n = 4)

L, linear effect of CIN dose; Q, quadratic effect of CIN dose

Variable	Diet	Treatment					s.e.m.	Statistical significance of the effects (P-value)			
		CON	CIN20	CIN60	CIN180	CIN540		CIN		Diet	CIN × diet
								L	Q		
pH	MC	6.63	6.62	6.65	6.65	6.64	0.028	0.37	0.86	<0.001	0.15
	HC	6.51	6.52	6.53	6.52	6.49	–	–	–	–	–
NH_3-N (mg/L)	MC	232	225	199[A]	188[A]	250[B]	10.0	<0.001	0.44	<0.001	0.04
	HC	95	95	87	78[B]	102	–	–	–	–	–
Total lactate (mg/L)	MC	13.0	10.8	12.3	12.1	292.5[A]	49.3	0.99	0.99	0.08	0.04
	HC	9.9	9.1	10.8	8.8	103.9[A]	–	–	–	–	–
Gas (mmol)	MC	3.22	3.25	3.26	3.22	3.12[A]	0.048	0.60	0.93	0.008	<0.001
	HC	3.35	3.33	3.34	3.39	2.96[A]	–	–	–	–	–
Methane (mmol)	MC	0.54	0.54	0.51	0.49	0.37[A]	0.031	0.17	0.83	<0.001	<0.001
	HC	0.47	0.46	0.47	0.47	0.11[A]	–	–	–	–	–
Methane/VFA (mol/mol)	MC	0.26	0.26	0.24	0.23	0.20[A]	0.018	0.10	0.51	<0.001	0.009
	HC	0.21	0.21	0.22	0.21	0.09[A]	–	–	–	–	–
Proportion of methane (mmol/mol gas)	MC	168	165	155	150	120[A]	9.0	0.12	0.83	<0.001	<0.001
	HC	140	139	144	139	38[A]	–	–	–	–	–
Hydrogen recovery (%)	MC	90.3	90.3	86.7	81.4[A]	79.7[A]	4.02	0.04	0.30	0.99	0.002
	HC	92.2	91.8	92.5	89.0	62.8[A]	–	–	–	–	–

[A]For each diet and variable, means differ from CON ($P < 0.05$).
[B]For each diet and variable, means differ from CON ($P < 0.10$).

that GO supplementation at 300 mg/L increased the proportion of propionate and butyrate and reduced acetate proportion. In contrast, Klevenhusen *et al.* (2011*b*) reported no effect of GO on VFA profile in the ruminal fluid from sheep fed a 50 : 50 forage : concentrate diet and supplemented daily with 5 g of GO for 19 days. Concentrations of GO in the study of Klevenhusen *et al.* (2011*b*), estimated assuming a mean ruminal volume of 7 L for a sheep (Ranilla *et al.* 1998), were ~736 mg/L, and even at this concentration, GO did not affect total VFA concentrations in the rumen or diet digestibility. It should be noticed that in sheep digesta outflow from the rumen would contribute to decrease ruminal GO concentrations over time, whereas there was no digesta outflow in the batch cultures. In our study, the amount of AFOM was not affected by any dose of GO with any diet type. The lack of effects of GO on total VFA production and AFOM indicates that GO did not modify overall diets fermentability, which agrees well with the results from the above cited studies.

Several *in vitro* studies have suggested that the effects of essential oils are pH-dependent, and this seems to be true for GO and garlic-related compounds (Cardozo *et al.* 2005; Kamel *et al.* 2008). Cardozo *et al.* (2005) found that GO had a more pronounced impact on rumen VFA profile at low compared with high rumen pH (5.5 versus 7.0), and proposed that the status of the molecules (i.e. dissociated or undissociated) may be dependent on rumen pH. However, batch cultures are usually highly buffered to enable ruminal microorganisms to grow for several hours without removal of fermentation products. In our study final pH in the cultures moved in a narrow range for both diets (6.52–6.67), although initial pH in the incubation media was higher for MC compared with HC diet (6.98 and 6.59, respectively). These results seem to preclude any influence of pH on the different effectiveness of GO observed for the two diets, and highlights the influence of the diet type on the observed differences.

Previous studies have reported significant methane suppressing effects of GO or its compounds (diallyl disulfide and allicin) supplemented at levels of 300 mg/L in batch cultures (Busquet *et al.* 2005*b*) and Rusitec fermenters (Soliva *et al.* 2011) or at 76 mg/L in batch cultures (Macheboeuf *et al.* 2006). Extracts of garlic bulbs or even garlic bulbs mixed with the diet have been shown to reduce methane production in batch cultures by Patra *et al.* (2010) and Staerfl *et al.* (2010), respectively. In contrast, no effects on methane production were observed by Kamel *et al.* (2008) with low doses of allicin and diallyl disulfide (up to 10 mg/L) and Kongmun *et al.* (2010) with garlic power at 320 mg/L. Only a few *in vivo* trials have investigated the effects of GO or its compounds on methane production, but their results are contrasting. Klevenhusen *et al.* (2011*a*) reported a decrease in methane production when sheep fed a 50 : 50 forage : concentrate diet were supplemented with diallyl disulfide (4 g per day), but no effects were shown with lower doses of diallyl disulfide (2 g) or 5 g of GO per day (Klevenhusen *et al.* 2011*b*). No effects on methane production were found when supplementing diallyl disulfide to lactating cows (3.3 g per day; van Zijderveld *et al.* 2011) or garlic bulbs (1% of DM intake) to sheep (Patra *et al.* 2011) and fattening bulls (Staerfl *et al.* 2012). Kongmun *et al.* (2011) observed that supplementing swamp buffalo with a mixture of 7% coconut oil and 100 g of garlic power reduced methane production by 9.1%, but a similar reduction was observed only with 7% coconut oil, which would indicate that the depressing effect on methane production was due to coconut oil rather than to garlic power. It is worth to mention

again that no dose of GO negatively affected overall diets fermentability, and therefore the methane : VFA ratio was decreased by GO60, GO180 and GO540, which would indicate a higher supply of energy for the host animal.

Hydrogen recovery, calculated from the stoichiometric relationships between the end products formed, was significantly lowered by GO180 and GO540 for both diets. This would indicate accumulation of hydrogen or a reduced end product other than methane, propionate, butyrate and valerate, since these are involved in the calculation of recovered hydrogen (Demeyer 1991). The lower hydrogen recovery found in our study does support the toxicity of organosulfur compounds from GO on ruminal methanogens, as previously indicated by others (Martin *et al.* 2010).

Cinnamaldehyde effects

At doses of 20, 60 and 180 mg/L, CIN had only subtle effects on *in vitro* ruminal fermentation. Compared with CON, CIN180 significantly increased acetate proportion by 2.4 and 2.9% for MC and HC diets, respectively, without affecting the proportions of any other VFA or total VFA production. This was reflected in a significant increase in acetate : propionate ratio in the CIN180-supplemented cultures for MC diet, but no changes were detected for HC diet. The minor changes in VFA proportions produced by CIN supplementation up to 180 mg/L did not result in differences in the amount of AFOM for any diet type, indicating no effect on diet fermentability. Similarly, Macheboeuf *et al.* (2008) reported that supplying CIN at 132 or 264 mg/L in batch cultures with a HC diet did not alter total VFA production.

Supplementing CIN540 significantly reduced total VFA production to 90 and 58% of CON for MC and HC diets, respectively. Furthermore, CIN540 reduced acetate proportion for MC diet, but increased acetate and reduced propionate proportions for HC diet, indicating a differential effect of CIN on VFA profile for the two diets. This is also indicated by the different response observed to CIN540 in acetate : propionate ratio, which was significantly decreased for MC diet, but increased for HC diet. These results would indicate some inhibition of fermentation activity when CIN was supplemented at 540 mg/L, being more pronounced for HC diet than for MC diet. This hypothesis is further supported by the lower gas (97 and 88% of CON values for MC and HC diets, respectively), and methane (69 and 24% of CON values for MC and HC diets, respectively), production. Moreover, lactate concentrations in CIN540 cultures were 23 and 10 times higher compared with those in CON cultures for MC and HC diets, respectively, which might be due to an inhibition of lactate utilisers, thus resulting in lactate accumulation. In agreement with our results, Macheboeuf *et al.* (2008) reported that supplying CIN at 396 mg/L in batch cultures with a 75 : 25 forage : concentrate diet decreased production of methane by 19% and VFA by 13%. In the study of Macheboeuf *et al.* (2008), CIN at 661 mg/L almost completely inhibited methane production (−94%) and dramatically reduced total VFA (−60%). Such changes indicate that, at high doses, the antimicrobial activity of CIN is sufficient to almost completely inhibit rumen microbial fermentation.

In our study, CIN60 and CIN180 significantly decreased NH_3-N concentrations compared with CON cultures for MC diet, but for HC diet only a trend of CIN180 to decrease NH_3-N concentrations was observed. The effects of CIN on rumen NH_3-N concentrations are controversial, and seem to be dose- and diet-dependent. Busquet and co-workers reported that CIN (98% purity) reduced NH_3-N concentrations by 12% in dual-flow continuous fermenters fed a HC diet and supplemented with 312 mg of CIN/L (Busquet *et al.* 2005*a*), and by 9.3% in batch cultures with a 50 : 50 forage : concentrate diet and supplemented with 310 mg of CIN/L (Busquet *et al.* 2006). Cardozo *et al.* (2005) reported reductions of 67 and 46% in the NH_3-N concentrations when supplementing CIN at 300 mg/L to batch cultures with a 10 : 90 forage : concentrate diet at pH = 7.0 or 5.5, respectively. In contrast, Benchaar *et al.* (2007) reported no effects of supplementing CIN at 400 mg/L to batch cultures of rumen fluid with a 51 : 49 forage : concentrate diet. In our study, CIN60 and CIN180 reduced NH_3-N concentrations by 14 and 19%, respectively, with MC diet, and by 8.4 and 18% for HC diet, which agrees well with the results of Busquet *et al.* (2005*a*, 2006). On the contrary, Chaves *et al.* (2008) found no effect of CIN on rumen NH_3-N concentrations in lambs fed a barley grain-based diet (700 g barley/kg of DM) and supplemented daily with 258 mg of CIN (86 mg/L, assuming a rumen volume of 3 L in 24-kg bodyweight lambs; Chaves *et al.* 2008). No effects on rumen NH_3-N concentrations were also found by Yang *et al.* (2010) when supplementing CIN to beef steers fed a diet containing 800 of barley grain per kg of DM and supplemented daily with 400, 800 and 1600 mg of CIN (concentrations of 6.7, 13.3 and 26.7 mg/L, respectively, assuming a rumen volume of 60 L for 538-kg bodyweight steers; Yang *et al.* 2010). The lack of effects found by Chaves *et al.* (2008) and Yang *et al.* (2010) in ruminants fed a HC diet agrees with our results for CIN20 and CIN60 with HC diet, and suggests that higher doses of CIN are needed to modify rumen fermentation *in vivo*. However, in the study of Yang *et al.* (2010), the highest dose decreased nutrient intake and ruminal digestibility, especially NDF and feed N digestibility, which highlights the importance of investigating the potential anti-nutritional side-effects of feed additives.

Diet effects

Differences between the two diet types in fermentation parameters followed the pattern observed in the ruminal fluid obtained from sheep fed the same diets (see Table 2). Differences in VFA profile between diets were also maintained in the cultures supplemented with GO and CIN, although the magnitude of the differences was sometimes modified by additive addition, which indicates different shifts in rumen microbial fermentation after additive supplementation. The higher NH_3-N concentrations in the cultures with MC diet compared with those in HC-cultures were probably due to the higher crude protein content of MC diet. Our results agree with previous studies (Gómez *et al.* 2005; Martínez *et al.* 2010) showing that methane production *in vitro* was affected by the forage : concentrate ratio in both the diet of donor sheep and the incubated substrate. Methane production was ~1.2 times higher for MC compared with HC diet (averaged across experimental treatments). However, it has to be taken into account that the

in vitro cultures used in the present study were heavily buffered, and the pH decrease observed in animals fed HC diets could not be reproduced; therefore, the influence of pH on the efficacy of the tested additives was not accounted for in the present study. Although *in vitro* studies have some limitations, they constitute a useful tool to test a high number of experimental treatments before performing *in vivo* trials.

Conclusions

The effects of GO and CIN on *in vitro* fermentation of two diet types (dairy versus intensive fattening) were both dose- and diet-dependent. In general, more effects were observed for GO compared with CIN when supplemented at the same levels. Garlic oil had more marked effects on VFA proportions with dairy diet, whereas effects on methane production were achieved with lower GO doses for the fattening one. Supplementation of GO up to 540 mg/L did not have any detrimental effect on ruminal fermentation, but the same level of CIN inhibited VFA production for both diets. In conclusion, the effectiveness of GO and CIN to manipulate ruminal fermentation may depend on the characteristics of the diet fed to the animals, which highlights the importance of testing these additives with different diet types. These results would have important practical implications if they are confirmed *in vivo*, although it has to be taken into account that some large doses used in the present study would be impractical to use in ruminant feeding.

Acknowledgements

Funding was provided by the Spanish C.I.C.Y.T. (Project AGL2008-04707-C02-02 and Acción Integrada AIB2010NZ-00190). C. Saro gratefully acknowledges the receipt of a scholarship from the M.E.C. of Spain (AP2006-03049).

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